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# Receptor Tyrosine Kinase Expression in Human Bone Marrow Stromal Cells

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Bone marrow stromal cells (BMSCs) are a heterogeneous population of cells derived from colony-forming units-fibroblastic (CFU-Fs). These cells reside in the bone marrow cavity and are capable of differentiating into several cell phenotypes including osteoblasts, chondroblasts, hematopoiesis-supporting stromal cells, and adipocytes. However, the factors that regulate the proliferation and differentiation of the BMSC population are for the most part unknown. Since many members of the receptor tyrosine kinase (RTK) family have been shown to participate in growth control of various mesenchymal cell populations, in this study we examined the expression and function of RTKs in the BMSC population. Degenerate oligonucleotides corresponding to two conserved catalytic domains of the RTK family and RT-PCR were used initially to determine which RTKs are expressed in the human BMSC (hBMSC) system. After subcloning the amplification product generated from mRNA of a multicolony-derived hBMSC strain, PDGF receptor (β), EGF receptor, FGF receptor 1, and Axl were identified by DNA sequencing of 26 bacterial colonies. Furthermore, PDGF and EGF were found to enhance BMSC growth in a dose-dependent manner and to induce tyrosine phosphorylation of intracellular molecules, including the PDGF and EGF receptors themselves, demonstrating the functionality of these receptors. On the other hand, bFGF was found to have little effect on proliferation or tyrosine phosphorylation. Since single colony-derived hBMSC strains are known to vary from one colony to another in colony habit (growth rate and colony structure) and the ability to form bone in vivo, the expression levels of these RTKs were determined in 18 hBMSC clonal strains by semiquantitative RT-PCR and were found to vary from one clonal strain to another. While not absolutely predictive of the osteogenic capacity of individual clonal strains, on average, relatively high levels of PDGF-receptor were found in bone-forming strains, while on average, nonbone-forming strains had relatively high levels of EGF-receptor. Taken together, these results indicate that RTKs play a role in the control of hBMSC proliferation, and that the differential pattern of RTK expression may be useful in correlating the biochemical properties of individual clonal strains with their ability to produce bone in vivo. J. Cell. Physiol. 177:426–438, 1998. 

Bone marrow stromal cells (BMSCs) are nonhematopoietic cells residing in the marrow cavity. While they exhibit a number of fibroblastic features, they are distinct from fibroblasts found in other connective tissues. Furthermore, they lack characteristics of endothelial cells and macrophages (Castro-Malaspina et al., 1980; Wang et al., 1990). After extensive proliferation in vitro, the BMSC population retains the capacity to differentiate into at least four types of connective tissues: bone, cartilage, hematopoiesis-supporting stroma, and associated adipocytes (Friedenstein, 1990; Friedenstein et al., 1974; Owen, 1988). Thus, the BMSC population contains pluripotent cells capable of both extensive proliferation and differentiation into several cell phenotypes (Owen and Friedenstein, 1988). When single marrow stromal cells (colony-forming units-fibroblastic; CFU-Fs) are allowed to develop into individual BMSC colonies, the colonies and the cells of which they are composed have different morphologies and rates of proliferation. Human BMSC (hBMSC) strains derived from individual colonies also vary widely in

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their ability to form bone and the hematopoietic microenvironment after in vivo transplantation (Kuznetsov et al., 1997a). These data demonstrate the heterogeneity of the hBMSC population. However, the distinguishing biochemical markers of different subsets of hBMSCs have not yet been found. Likewise, the factors that regulate the proliferation of the different members of the hBMSC population still remain to be elucidated.

Receptor tyrosine kinases (RTKs) are growth factor receptors with intrinsic protein tyrosine kinase activity, and are thought to regulate cell proliferation and differentiation via intracellular signal transduction mechanisms (Fantle et al., 1993). RTKs possess a large glycosylated extracellular domain, a single hydrophobic transmembrane region, and a cytoplasmic domain that contains tyrosine kinase activity (Hanks et al., 1988). The binding of ligand to the extracellular domain of RTK promotes receptor dimerization or oligomerization, resulting in transphosphorylation and activation of the kinase domain. These receptors, in turn, initiate a variety of signaling cascades necessary for cell proliferation and differentiation (Heldin, 1995; Ullrich and Schlessinger, 1990). Consequently, RTKs are a potential gateway in the response of cells to stimulation by growth factors.

The first goal of this study was to amplify all RTK-related mRNA isolated from a multicolony-derived hBMSC strain and to begin to identify the RTK members that are present in this pluripotent population of cells. This was achieved by designing a set of degenerate oligonucleotide primers that recognize two highly conserved regions (PTK I and PTK II) of the catalytic domain of the receptor tyrosine kinases (Wilks, 1990). Our second goal was to confirm the functionality of several RTKs expressed in hBMSCs by examining cell proliferation after stimulation with specific growth factors, and tyrosine phosphorylation of intracellular proteins and receptors. The last goal was to begin to evaluate the pattern of RTK expression of individual hBMSC strains within the heterogeneous hBMSC population. For this purpose, the pattern of RTK expression in 18 single colony-derived hBMSC strains with varying growth patterns and differentiation potentials (as determined by an in vivo transplantation assay) was characterized by RT-PCR, using receptor-specific oligonucleotide primers.

## MATERIALS AND METHODS

### hBMSC isolation and culture

Fragments of bone with bone marrow tissue were received from patients undergoing corrective surgery, and bone marrow aspirates were from normal volunteers in accordance with NIH regulations governing the use of human subjects (Protocol #94-D-0188). Bone marrow from surgical specimens was scraped with a steel blade into ice-cold α-modified Minimum Essential Medium (αMEM, Life Technologies, Grand Island, NY) and vigorously pipetted. Bone marrow aspirates were collected into the same medium containing 100 U/ml of sodium heparin (Fisher Scientific, Fair Lawn, NJ) and vigorously pipetted. Cell suspensions were passed several times through 16- and 20-gauge needles and filtered through a cell strainer (Becton Dickinson, Franklin Lakes, NJ) to remove cell aggregates. Single-cell suspensions were plated at either high cell density (for multicolony-derived strains) or low cell density (for single colony-derived strains) in αMEM containing 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate (Biofluids, Rockville, MD), and 20% fetal bovine serum (FBS, Atlanta Biological, Norcross, GA). The cultures were maintained at 37°C in 5% $CO_2$ in air. Medium was changed on day 1 for aspirates and on day 7 and day 14 for all cultures if they were not passaged (Kuznetsov et al., 1997a).

Multicolony-derived hBMSC strains were prepared by plating bone marrow suspensions into 75-$cm^2$ plastic culture flasks (Becton Dickinson) at $1-3 \times 10^7$ nucleated cells per flask. When the hBMSC layer was close to confluency (10–14 days after plating), the cells were removed with trypsin-EDTA (Life Technologies) and replated at $2 \times 10^6$ cells per 75-$cm^2$ flask. The second passage was performed at 4–5 days after the first passage.

Single-colony derived hBMSC strains were prepared by plating bone marrow single-cell suspensions into 150-mm petri dishes (Becton Dickinson) at $1 \times 10^4-1 \times 10^5$ cells per dish. After 20 days of culture, discrete hBMSC colonies were removed individually with a cell scraper, transferred into microfuge tubes, and treated with trypsin-EDTA solution for 15–20 min at room temperature. Alternatively, individual colonies were treated with trypsin-EDTA using cloning cylinders. In both cases, the cells were then transferred into individual wells of six-well culture plates (Becton Dickinson) and cultured in the same medium. At approximately 70% confluency, hBMSCs were passaged consecutively into 25-$cm^2$ and then to 75-$cm^2$ flasks. For the studies reported here, two multicolony-derived cell populations from 2 different patients and 18 single colony-derived strains generated from 7 donors were used. Characteristics of these strains are shown in Table 1.

### Estimation of initial proliferation rate in single colony-derived hBMSCs

The cell number of newly formed colonies cannot be determined by direct cell counting after the colony contains more than 200–300 cells; nor can a standard growth curve be performed during the early passages (there are insufficient numbers of cells). However, an approximate rate of proliferation can be assessed by comparing two values: 1) the cell yield after the fourth passage, and 2) the time period it takes this particular cell number to be achieved. All strains were handled identically, and passaged to a larger vessel at the same state of confluence.

### RNA isolation, amplification of RTK mRNA, and subcloning

Total RNA was extracted from a subconfluent, second-passage multicolony-derived hBMSC strain using either $CsCl_2$ centrifugation or RNA STAT-60 (Teltest, Friendswood, TX). Degenerate oligonucleotide primers that spanned the conserved region of the RTK family were prepared to generate the cDNA-encoding RTK sequences, PTK I and PTK II (Fig. 1). RT-PCR was performed as described previously (Ibaraki et al., 1995), using the primers at a final concentration of 0.5 μmole/l. The size of the PCR product was analyzed on a 6% nondenaturing acrylamide gel (Novex, San Diego, CA) in Tris-borate-EDTA buffer (TBE).

TABLE 1. Characteristics of multicolony-derived and single colony-derived hBMSC strains

| Donor no. | Clone no. | Original BMSC colony | | | Proliferation rate[1] | BF in vivo[2] |
|---|---|---|---|---|---|---|
| | | Colony structure | Cell shape | Colony size | | |
| 1 | Multicolony-derived | | | | 22.4 | 3.5 |
| 2 | 1 | Multi-l nod | Spindle | Large | 2.6 | 1.0 |
| 3 | 2 | Multi-l nod | Spindle | Large | 3.0 | 0.0 |
| 4 | 3 | Multi-l nod | Spindle | Large | 0.8 | 2.5 |
| | 4 | One-layer | Flat | Small | 2.4 | 0.8 |
| | 5 | Multi-l nod | Spindle | Large | 11.8 | 0.0 |
| 5 | 6 | Multilayer | Spindle | Large | 2.1 (P5) | 0.0 |
| 6 | Multicolony-derived | | | | 26.2 | 3.6 |
| | 8 | Multi-l nod | Spindle | Large | 9.6 | 1.0 |
| | 7 | One-layer | Spindle | Large | 8.8 | 1.7 |
| | 11 | One-layer | Flat | Small | 3.2 | 0.0 |
| | 9 | One-layer | Intermediate | Average | 3.6 | 0.0 |
| | 10 | Multilayer | Flat | Large | 1.2 | 1.0 |
| | 12 | Multilayer | Flat | Large | 2.4 | 0.0 |
| 7 | 13 | Multi-l nod | Spindle | Large | 18.0 | 3.4 |
| | 14 | One-layer | Intermediate | Average | 11.2 | 2.7 |
| | 15 | Multilayer | Spindle | Average | 6.8 | 1.8 |
| | 16 | Multilayer | Spindle | Average | 7.2 | 0.0 |
| | 17 | Multi-l nod | Spindle | Large | 8.4 | 2.1 |
| | 18 | One-layer | Spindle | Average | 4.8 | 0.0 |

[1]Proliferation rate = cell number $\times 10^{-5}$/day, estimated from the number of cells at the fourth passage divided by the number of days it took to reach the fourth passage.
[2]BF, bone formation, determined by transplantation of BMSCs on hydroxyapatite-tricalcium phosphate particles into immunocompromised mice.

cDNA generated by RT-PCR, using the oligonucleotides for PTK I and PTK II, was purified and ligated to the pCR II vector using the ingredients in the TA cloning system (Invitrogen, Carlsbad, CA) following the manufacturer's recommendations. Twenty-six insert-containing bacterial colonies were randomly selected and sequenced using conventional procedures. The identity of the cDNA clones obtained was determined using the GCG Wisconsin computer program (Genetics Computer Group, Madison, WI).

### Cell proliferation assay

The effect of platelet derived growth factor-BB (PDGF-BB), epidermal growth factor (EGF), and basic fibroblast growth factor (bFGF) on cell proliferation was analyzed using a crystal violet staining method as described previously (Fedarko et al., 1995; Gronthos and Simmons, 1995). In brief, cells were seeded in 24-well culture plates at a cell density of $2 \times 10^4$ cells per well. After 24 hr, cells were rinsed with serum-free αMEM three times and then cultured in serum-free medium consisting of αMEM, 2 mM L-glutamine, $10^{-7}$ M dexamethasone (Sigma, St. Louis, MO), $10^{-4}$ M L-ascorbic acid phosphate magnesium salt n-hydrate (Wako Pure Chemical Industries, Osaka, Japan), 10 μg/ml bovine pancreatic insulin (Sigma), 2% bovine serum albumin, 4 μg/ml human low-density lipoprotein (Sigma), 200 μg/ml human transferrin (Sigma), $5 \times 10^{-5}$ M β-mercaptoethanol (Sigma), 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, and various concentrations of growth factors (0, 0.1, 1, 10, 50, and 100 ng/ml). After 7 days of culture, the dose response of the cells to the growth factors was determined by rinsing with phosphate-buffered saline (PBS) and fixing with 1% glutaraldehyde in PBS for 15 min. Fixed cells were stored in PBS at 4°C until the end of the experiment, when all plates were processed simultaneously. Cells were stained by incubation with 0.02% crystal violet in deionized water for 30 min at room temperature. The cells were rinsed twice with deionized water, and the stain bound to cells was extracted by overnight incubation with 300 μl/well of 70% ethanol. Absorbency was measured at 578 nm, using a Spectramax 340 microplate reader (Molecular Devices, Sunnyvale, CA).

To determine the effect of growth factors with time in culture, cells were cultured as described above except that the medium contained 10 ng/ml of growth factors. At different intervals between days 0–14 of culture, cells were rinsed, fixed, and stained, and the absorbency was measured at 578 nm as described above.

### Analysis of intracellular tyrosine-phosphorylated proteins

The functionality of PDGF and EGF receptors was determined by examining the tyrosine phosphorylation of intracellular protein after stimulation with growth factors by Western blot analysis. The cells of a multicolony-derived population and two single colony-derived strains (a bone-forming strain, clone 1, and a nonbone-forming strain, clone 2) were cultured in 60-mm dishes (Becton Dickinson). At 80–90% of confluency, the medium was replaced with serum-free αMEM for 24 hr. The cells were stimulated with 100 ng/ml of recombinant human EGF and PDGF-BB (R&D Systems, Minneapolis, MN) for 5 min at 37°C, and then lysed in 200 μl of a modified RIPA lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM sodium chloride, 0.1% sodium deoxycholate, 1 mM EDTA, 1% NP-40, 0.1% SDS, 1 mM sodium orthovanadate, 1 mM polymethylsulfonyl fluoride, 20 μg/ml aprotinin, and 20 μg/ml leupeptin) on ice. After centrifugation of the lysate at 14,000*g* for 5 min, the supernatant was added with the same volume of 2× Laemmli sample buffer and boiled for 3 min. The proteins were separated on 4–20% SDS-PAGE (Novex, San Diego, CA) and transblotted to nitrocellulose membrane. The membrane was incubated overnight with horseradish peroxidase-conjugated anti-phosphotyrosine monoclonal antibody 4G10 (Upstate, Lake Placid, NY). The immunocomplexes were detected by an en-

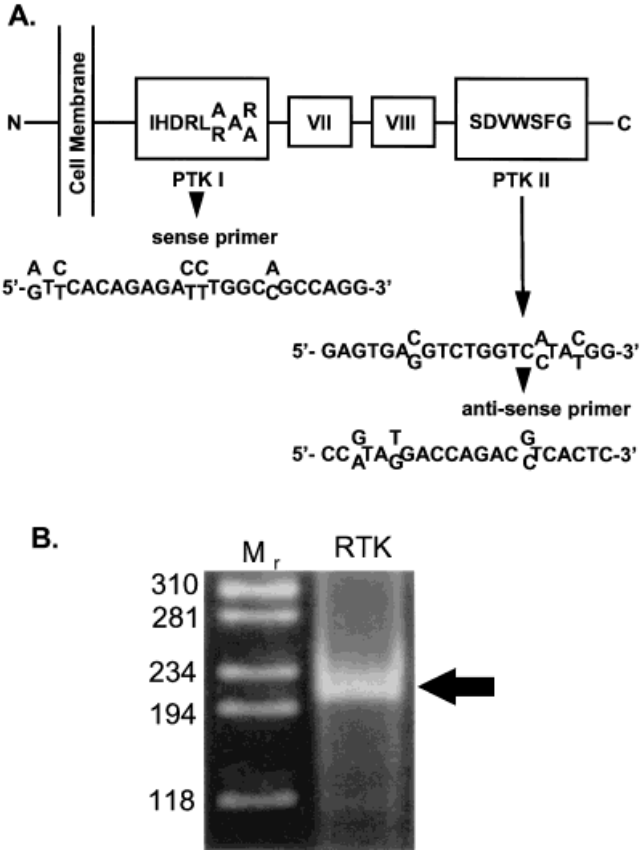


Fig. 1. Conserved catalytic domains of the protein tyrosine kinase family, and PCR amplification from mRNA of hBMSCs. **A:** Conserved sequence motifs of the intracellular domains of the RTK family are indicated by Roman numerals, with amino acid sequences indicated by conventional amino acid single letter code. Position and sequence of the degenerate oligonucleotides used for the PCR reaction are indicated. **B:** Polymerase chain reaction product from mRNA derived from a multicolony-derived preparation of human marrow stromal fibroblasts. $M_r$, molecular marker; RTK, PCR product.

hanced chemiluminescence assay (ECL, Amersham Life Science, Buckinghamshire, UK).

### Immunoprecipitation of receptor tyrosine kinases

Immunoprecipitation was performed using an antibody against PDGF-R (β) and EGF-R in order to demonstrate tyrosine phosphorylation of each receptor. The cells of a multicolony-derived population and two single colony-derived strains (a bone-forming strain, clone 1, and a nonbone-forming strain, clone 2) were stimulated with growth factors, as described above. The cells were lysed in 400 μl of the same lysis buffer on ice. The lysate was centrifuged at 14,000*g* for 5 min, and the supernatant was incubated with 20 μl of protein G-Sepharose (Pierce, Rockford, IL) for 1 hr to remove material that adsorbs nonspecifically to this resin. After centrifugation, the supernatant was transferred to a new tube, and 2 μg of anti-human PDGF receptor (β) IgG (Genzyme Corporation, Cambridge, MA) or anti-human EGF receptor IgG (Upstate) antibody and 20 μl of protein G-Sepharose were added. Following an overnight incubation with gentle agitation at 4°C, the immunoprecipitate was washed four times with lysis buffer. The immunocomplexes were separated by 4–20% SDS-PAGE and transferred to nitrocellulose membrane. The membrane was incubated overnight with horseradish peroxidase-conjugated anti-phosphotyrosine monoclonal antibody 4G10. The immunoreactive bands were detected by the ECL system. After stripping off the antibody, the same blot was incubated overnight with anti-human PDGF receptor (β) or anti-human EGF receptor antibody, and then incubated with a horseradish peroxidase-labeled anti-mouse IgG antibody (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). Immunocomplexes were detected by the same ECL system.

### Expression of RTK mRNA in single colony-derived hBMSCs

Total RNA was isolated from two multicolony-derived strains at the second passage, and from 18 single colony-derived hBMSC populations at the fourth or fifth passage, using RNA STAT-60 (Teltest). At this point, approximately half of the cells were used for RNA extraction and the other half were used for the in vivo assay of osteogenesis as described below. Although a series of 34 clonally derived strains had been studied previously (Kuznetsov et al., 1997b), not all strains could be utilized in this study since many had stopped proliferating and did not yield enough cells for mRNA preparation. The characteristics of the 18 clones are indicated in Table 1. Because only a limited number of cells can be generated from a single colony, it was not possible to isolate enough RNA for Northern blotting using specific RTK probes. Consequently, the level of mRNA for individual RTKs was determined by semiquantitative RT-PCR, using the primer sets indicated in Table 2. Total RNA (2–5 μg) from the multicolony-derived strains and from each clonal strain was incubated for 15 min at room temperature (RT) with DNAse I (GIBCO BRL, Rockville, MD) to avoid any possible contamination of genomic DNA. RNA was then reverse-transcribed into cDNA (Perkin-Elmer Cetus, Foster City, CA, or GIBCO BRL Preamplification System for first-strand cDNA synthesis), and PCR was performed using oligonucleotide primers specific for the individual RTKs included in this study (Table 2) at a final concentration of 0.5 μmole/l, using a commercial amplification buffer (Master Mix, Boehringer Mannheim, Indianapolis, IN). RT-PCR products were analyzed on 6% nondenaturing polyacrylamide gels and visualized either by staining with ethidium bromide or by staining for 30 min with a highly sensitive fluorescent stain (Syber Green I, FMC Bio Products, Natick, MA) according to the manufacturer's directions. Using an optical scanner and IMAGEQUANT software (Molecular Dynamics, Sunnyvale, CA), quantitation of the signal was obtained. A relative mRNA expression value for RTKs was estimated after normalization against either GapdH or osteonectin (the expression of which does not change from one colony to another, data not shown), providing a semiquantitative analysis of the data. Experiments were performed at least twice for each RTK; in each case, the same relative patterns of RTK were observed.

TABLE 2. Primers used in RT-PCR for specific RTKs

PDGF-R(β)
Sense: 5′-(3296) TGACCACCCAGCCATCCTTC(3315)-3′
Antisense: 5′-(3523)GAGGAGGTGTTGACTTCATTC(3503)-3′
Amplified fragment: 228 bp
EGF-R
Sense: 5′-(2864)ACCAGAGTGATGTCTGGAGC(2883)-3′
Antisense: 5′-(3240)GATGAGGTACTCGTCGGCAT(3221)-3′
Amplified fragment: 377 bp
FGF-R1
Sense: 5′-(1591)AAGGACAAACCCAACCGTGTGACC(1614)-3′
Antisense: 5′-(2016)GCCAAAGTCTGCTATCTTCATCAC(1993)-3′
Amplified fragment: 426 bp
Axl
Sense: 5′-(1820)GGTGGCTGTGAAGACGATGA(1839)-3′
Antisense: 5′-(2122)CTCAGATACTCCATGCCACT(2103)-3′
Amplified fragment: 303 bp
Osteonectin
Sense: 5′-(58)ATGAGGGCCTGGATCTTCTT(77)-3′
Antisense: 5′-(460)TGCCCTCCAGGGTGCACTT(442)-3′
Amplified fragment: 403 bp

### Cell transplantation assay

Cells derived from a multicolony-derived population and 18 single colony-derived strains were transplanted into immunocompromised mice in order to determine the capacity of these strains to form bone in vivo, as described previously (Krebsbach et al., 1997; Kuznetsov et al., 1997b). Sixteen of the 18 clonal strains used in this study have been partially described previously (Kuznetsov et al., 1997b).

Briefly, $1.0-3.0 \times 10^6$ cells were incubated with 40 mg of hydroxyapatite-tricalcium phosphate particles (Zimmer, Warsaw, IN) in 1 ml of medium at 37°C for 70–100 min with slow rotation (25 rpm). The particles with adherent cells were collected by brief centrifugation and transplanted subcutaneously into 8–10-week-old immunocompromised female mice (NIH-bg-nuxidBR, Harlan Sprague Dawley, Indianapolis, IN). The operation was performed in accordance with the specifications of an approved NIH small animal protocol (#114-93) under anesthesia achieved by intraperitoneal injection of 2.5% avertin at 18 ml/g of body weight. Midlongitudinal skin incisions of about 1 cm in length were made on the dorsal surface of each mouse, and subcutaneous pockets were formed by blunt dissection. A single transplant was placed into each pocket, with up to four transplants per animal. The incisions were closed with surgical staples. Two transplants per strain were examined.

### Characterization of tissue formed by transplants of single colony-derived BMSCs

The transplants were retrieved at 8 weeks after transplantation. Each transplant was cut into 2–4 pieces, fixed, and partially decalcified for 2 days in Bouin's solution (Sigma). After dehydration with an ascending series of ethanol and xylene, the transplants were embedded in paraffin. Sections 5 μm in thickness were stained with hematoxylin and eosin, and observed microscopically. The amount of bone formed in each transplant was evaluated on a scale of 0–4 by four independent observers.

Verification that the bone and stroma formed in the ossicles were of human origin was determined by immunohistochemistry and in situ hybridization techniques, as described previously (Kuznetsov et al., 1997b). In brief, rabbit anti-human osteonectin antibody (HON), which does not crossreact with mouse osteonectin, was kindly provided by Dr. Larry W. Fisher (Craniofacial Skeletal Diseases Branch, National Institute of Dental Research, NIH, Bethesda, MD) (Fisher et al., 1995). After deparaffinization and rehydration, the sections were incubated in 0.13% pepsin/0.01 N HCl for 1 hr at 37°C to reactivate the antigenicity of osteonectin. Indirect immunohistochemistry was carried out using HON (1:100) as primary antibody and horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:200, Kirkegaard & Perry Laboratories) as secondary antibody. Mouse vertebral bone and human femoral bone were used for negative and positive control, respectively. As another negative control, the primary antibody was substituted for normal rabbit serum (Vector Laboratories, Burlingame, CA).

In situ hybridization using the human-specific *alu* sequence was also used to confirm the origin of newly formed bone. A digoxigenin-labeled probe specific for the human *alu* sequence was prepared by PCR, using human genomic DNA and specific primers (sense, 5′-GTGGCTCACGCCTGTAATCC-3′; antisense, 5′-TTTTTTGAGACGGAGTCTCGC-3′). Deparaffinized sections were immersed in 0.2 N HCl at room temperature for 7 min. After washing with PBS, the sections were treated with 1 mg/ml pepsin in 0.01 N HCl at 37°C for 10 min and with 0.25% acetic acid/0.1 M triethanolamine (pH 8.0) at room temperature for 10 min, and prehybridized with 50% deionized formamide/4× SSC at 37°C for 15 min. The sections were then hybridized with 5 ng/ml digoxigenin-labeled probe in hybridization buffer (1× Denhardt's solution, 5% dextran sulfate, 0.2 mg/ml salmon sperm DNA, 4× SSC, and 50% deionized formamide) at 42°C for 3 hr after a denaturation step at 95°C for 3 min. After washing with 2× SSC at room temperature and 0.1× SSC at 42°C, digoxigenin-labeled DNA was detected by immunohistochemistry, using anti-digoxigenin alkaline phosphatase-conjugated Fab fragments (Boehringer Mannheim, Indianapolis, IN).

### Comparison of RTK expression to osteogenic capacity of single colony-derived hBMSC strains

Two methods were used to statistically analyze RTK levels. In the first, clonal strains were grouped as either "bone-forming" or "nonbone-forming," based upon results of the in vivo assay system. Because of the limited number of clonal strains analyzed (10 in the bone-forming group and 8 in the nonbone-forming group) and the inherent variability from one strain to another, the values for RTK mRNA levels normalized to either GapdH or osteonectin were expressed as a percent distribution among the four RTKs studied here, and analyzed nonparametrically using Wilcoxon's sum of rank test. Thus, the distribution of the data, and not the means, was compared between the two groups for each RTK. Statistical significance was indicated by $P < 0.05$. In the second method of statistical analysis, the amount of bone formed by the bone-forming strains was subjected to linear regression analysis against proliferative capacity, to ascertain whether the amount of bone formed was related to the proliferative capacity of those strains.

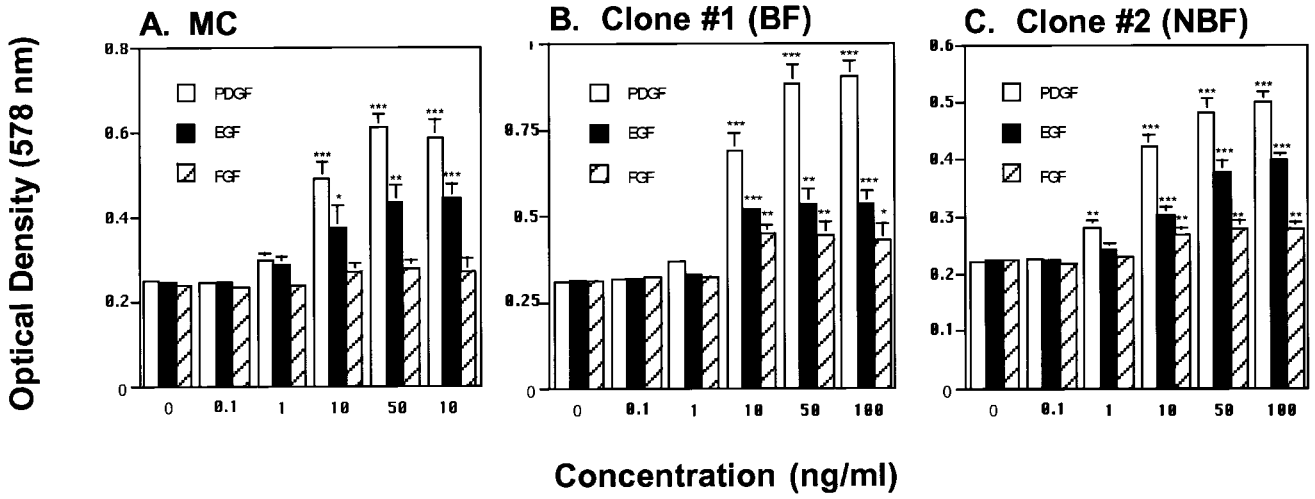


Fig. 2. Effect of PDGF-BB, EGF, and bFGF on proliferation of hBMSCs. Growth factors were added in increasing concentration to subconfluent cultures of a multicolony-derived strain (**A**) and two single colony-derived strains **B,** a bone-forming strain, clone 1; **C,** a nonbone-forming strain, clone 2). After 7 days, the cells were fixed with 1% glutaraldehyde, and stained with 0.02% crystal violet. The dye was eluted with 70% ethanol, and absorbency was measured at 578 nm. Values are the mean ± SD from three wells in a representative experiment. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. the culture without growth factor.

## RESULTS

### RTK expression in multicolony-derived hBMSCs

When total RNA from multicolony-derived hBMSCs was amplified using a degenerate oligonucleotide primer set spanning the PTK I and PTK II regions (Fig. 1A), a single relatively broad band of approximately 200 bp was observed (Fig. 1B). This length is characteristic for the members of the RTK family described thus far (Heldin, 1995). The nature of the products contained within this band was determined by subcloning and sequencing. Twenty-six bacterial colonies were randomly selected and were shown by DNA sequence analysis to be identical to the following RTKs: platelet-derived growth factor receptor (PDGF-R, β isoform), epidermal growth factor receptor (EGF-R), fibroblast growth factor receptor 1 (FGF-R1), and the newly described receptor, Axl. Of the clones sequenced, 11 were PDGF-R, 8 were EGF-R, 2 were FGF-R1, and 5 were Axl.

### Growth factor stimulation of multicolony-derived-hBMSC, proliferation, and receptor phosphorylation

Based on the identification of the receptors for PDGF-BB, EGF, and bFGF, experiments were designed to investigate the effect of these growth factors on the in vitro proliferation of multicolony-derived BMSCs. Subconfluent cells were stimulated via their RTKs, using increasing concentrations of specific growth factors (PDGF-BB, EGF, and bFGF). The proliferation of multicolony-derived BMSCs was stimulated by PDGF-BB and EGF at concentrations equal to or greater than 10 ng/ml. On the other hand, bFGF did not have a marked effect on proliferation even at higher doses, and was not studied further (Fig. 2A). These results indicate that PDGF-BB is a more potent stimulator of multicolony-derived hBMSC proliferation compared to EGF, which has a moderate effect on the proliferation of multicolony-derived hBMSCs.

We next investigated whether PDGF-BB and EGF were able to induce intracellular tyrosine phosphorylation via their RTKs. Cells were treated with 100 ng/ml of PDGF-BB and EGF for 5 min. Western blot analysis of intracellular molecules with anti-phosphotyrosine antibody showed that treatment with PDGF-BB and EGF was able to induce tyrosine phosphorylation of intracellular molecules in multicolony-derived hBMSCs (Fig. 3A). In addition, the cell lysates were immunoprecipitated with anti-PDGF-R (β) or anti-EGF-R and then analyzed by Western blotting, using anti-phosphotyrosine antibody. The 180-kDa PDGF-R (Fig. 4A) and 175-kDa EGF-R (Fig. 4B) were tyrosine-phosphorylated in response to the stimulation with each ligand. The levels of PDGF-R and EGF-R are shown in Figure 4A,B at bottom, indicating that the level of tyrosine phosphorylation of RTK was increased independent of an increase in the amount of receptor.

### Heterogeneity of hBMSC colonies

It is well-recognized that when single-cell suspensions of bone marrow are plated at low density, there is heterogeneity in the types of colonies that are formed (Fig. 5A). At 20 days after explantation, small colonies (2 colonies) contained no more than 200 cells, average-sized colonies (5 colonies) consisted of several hundred to one thousand hBMSCs, and large colonies (11 colonies) contained several thousand cells. Twelve hBMSC colonies had a multilayered structure and the remaining 6 were monolayered. Of the 12 multilayered colonies, 7 also contained nodular structures. In other experiments, these types of structures were found to be alizarin red-positive, indicative of calcium accumulation (data not shown). The colonies also differed in

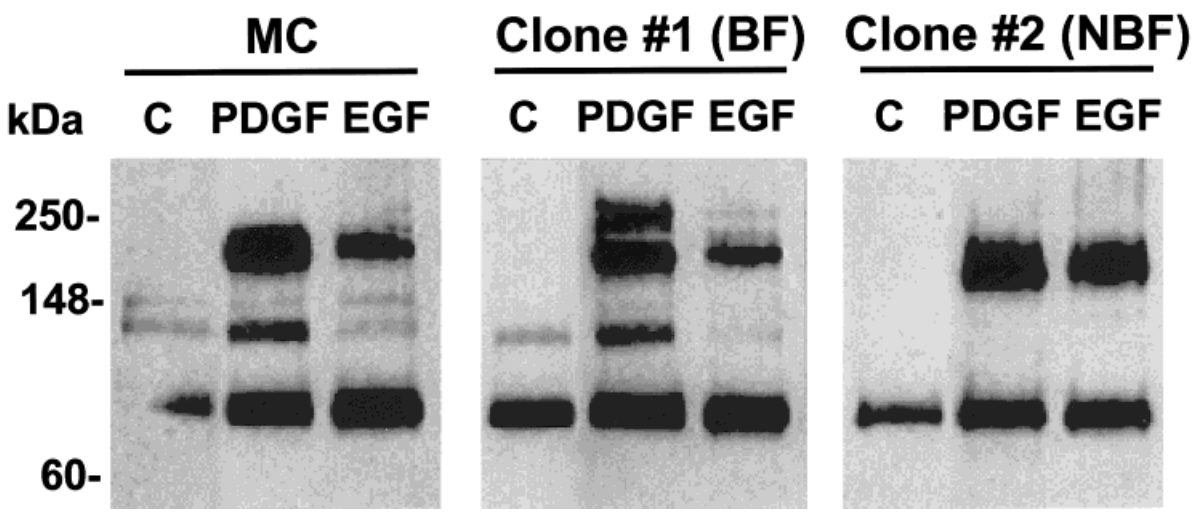


Fig. 3. Effect of PDGF-BB and EGF on intracellular tyrosine phosphorylation in hBMSCs. A multicolony-derived strain (MC) and two single colony-derived strains (a bone-forming strain, clone 1, and a nonbone-forming strain, clone 2) were treated without (C) or with 100 ng/ml of each growth factor for 5 min. The cell lysate was subjected to SDS-PAGE and the immunoblots were incubated with monoclonal anti-phosphotyrosine antibody (4G10).

cell morphology, ranging from spindle-shaped cells (12 colonies) to an intermediate shape (2 colonies) to large, flattened cells (4 colonies) (Fig. 5B). All multilayered, nodular colonies were composed of spindle-shaped cells, whereas other multilayered colonies contained either spindle-shaped or flat cells. Intermediate-shaped cells were found in monolayered colonies. These results indicate that the rate of proliferation of each CFU-F is variable (based on the number of cells within a colony), and results in the formation of colonies with varying sizes and cell densities. The characteristics of the strains derived from different colonies are described in Table 1.

### Expression of RTK mRNA in single colony-derived BMSC strains

The relative proportion of RTK mRNA in hBMSC clonal strains was analyzed to assess if the pattern of expression could be related to the differences in colony growth habits noted in low-density cultures of hBMSCs. RNA harvested from 18 different clonal strains was amplified by RT-PCR, using oligonucleotide primers specific for PDGF-R, EGF-R, FGF-R, and Axl (Table 3). The data indicate that there is variability in the pattern of expression from one strain to another, although repeated analysis of the same strain gave the same results. When looking at the level of expression as the percent distribution of the four RTKs studied here, Axl was, in general, the most abundant RTK in all hBMSC strains, followed by PDGF-R. EGF-R and FGF-R expression was also variable, but generally expressed at lower levels than Axl and PDGF-R. Furthermore, there was no consistent pattern of RTK expression that was indicative of a particular type of clonal strain (i.e., monolayer vs. multilayer; flat vs. intermediate vs. spindle-shaped cells; or nodular vs. nonnodular).

### Effect of growth factors on single colony-derived hBMSCs

The effects of PDGF-BB, EGF, and bFGF on proliferation and tyrosine phosphorylation of two single colony-derived hBMSCs were determined. A bone-forming strain (clone 1) was stimulated by both growth factors (PDGF and EGF) at concentrations starting at 10 ng/ml (Fig. 2B). The proliferation of a nonbone-forming strain (clone 2) was also stimulated by PDGF at concentrations equal to or greater than 1 ng/ml, and by EGF at concentrations equal to and greater than 10 ng/ml (Fig. 2C). Proliferation of neither clonal strain was significantly stimulated by increasing concentrations of bFGF, and consequently, bFGF was not studied further. Growth curve analysis using these growth factors at the concentration of 10 ng/ml showed that PDGF and EGF enhanced cell proliferation of both bone- and nonbone-forming clonal strains (Fig. 6B,C). PDGF-BB was more active in stimulating proliferation than EGF in the bone-forming strain (clone 1) compared to the nonbone-forming strain (clone 2), whereas PDGF-BB and EGF were nearly equivalent in stimulating proliferation in the dose-response study in the nonbone-forming strain (clone 2) (compare the y-axes in Fig. 2 and Fig. 6B,C).

In addition, both growth factors stimulated intracellular tyrosine phosphorylation in both of the single colony-derived strains (clone 1 and clone 2) (Fig. 3B,C), and of PDGF-R (β) and EGF-R (Fig. 4A,B). The pattern of intracellular molecules that became tyrosine-phosphorylated after treatment of the bone-forming strain (clone 1) with PDGF-BB was different compared to the pattern stimulated by EGF, and both of these patterns were different compared to the pattern elicited by both PDGF-BB and EGF in the nonbone-forming strain (clone 2) (Fig. 3B,C). These data strongly support the

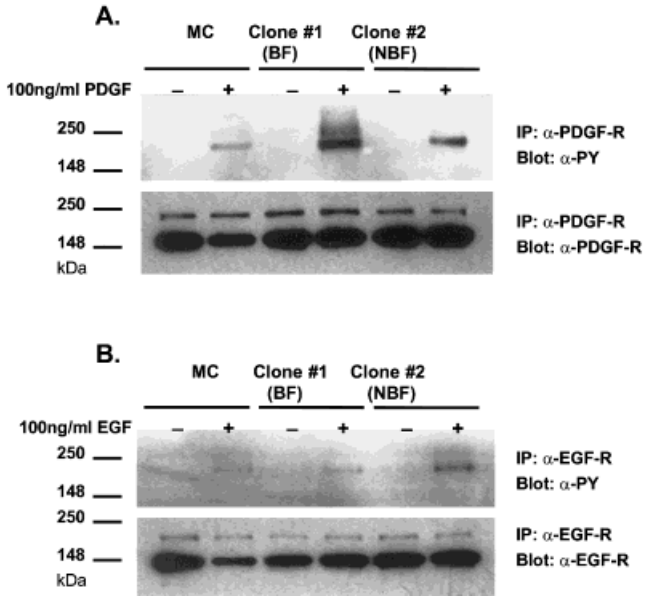


Fig. 4. Immunoprecipitation of RTKs from hBMSCs after treatment with PDGF-BB and EGF. Cell extracts of a multicolony-derived strain (MC) and two single colony-derived strains (a bone-forming strain, clone 1, and a nonbone-forming strain, clone 2) were immunoprecipitated with anti-PDGF-R (**A**) or anti-EGF-R (**B**) antibody. Each cell strain was treated without (−) or with 100 ng/ml of PDGF-BB or EGF for 5 min. After immunoprecipitation (IP) with anti-PDGF-R (β) (A) or anti-EGF-R (B), Western blot analysis (Blot) of the immunoprecipitates was performed with monoclonal anti-phosphotyrosine antibody (4G10) (**above**) or monoclonal antibody against each growth factor receptor (**below**). Some of the major bands shown in the lower panels are mouse IgG heavy chains.

suggestion that RTKs may differentially regulate metabolic activity of different members of the BMSC family.

### Bone formation by in vivo transplantation of hBMSCs

Substantial amounts of new bone (3−4 on a scale of 0 to 4+) were formed in the transplants of the multicolony-derived strains. In contrast, of the 18 single colony-derived strains used in this study, 10 formed bone ranging from barely detectable levels (0.8 on a scale of 0 to 4+) up to levels equivalent to the amount formed by multicolony-derived strains. Interestingly, some clonal strains that were multilayered and formed nodules in culture were not able to support bone formation in vivo, while other colonies that did not multilayer, or form nodules, did form bone (Table 1).

To confirm the origin of the cells of the newly formed bone, immunohistochemistry using human osteonectin-specific antibody, HON, and in situ hybridization using a specific probe for human repetitive *alu* sequence were performed. Cells of newly formed bone demonstrated intense immunostaining with anti-human osteonectin antibody (Fig. 7B,E,H). No immunoreactivity above background levels was seen in mouse tissue surrounding the transplants (data not shown). Also, in situ hybridization with human-specific *alu* probe showed that the cells in bone tissue and some stromal cells within the transplant were positive for human-specific repetitive *alu* sequence (Fig. 7C,F,I). *Alu* sequences were not detected in hematopoietic cells in the transplants, indicating the recipient origin of hematopoietic cells, as reported previously (Kuznetsov et al., 1997b).

### Comparison of RTK expression to bone formation in single colony-derived hBMSCs

Extensive statistical analysis of the data indicates that there is no single parameter, or even sets of parameters (including cell morphology, colony size or growth habit, and pattern of RTK expression), that can be considered predictive of the ability of hBMSCs to form bone in vivo. Nevertheless, on average, bone-forming strains had relatively higher levels of PDGF-R and relatively lower levels of EGF-R than nonbone-forming strains, but again, there was variability in the levels of these two receptors within both bone-forming and nonbone-forming strains. In addition, in bone-forming strains, the higher the rate of proliferation, the more bone was formed. However, the proliferation rate was not predictive of osteogenesis, as some fast-growing strains did not make bone (see Table 3 and Fig. 8).

## DISCUSSION

Bone marrow contains nonhematopoietic stromal cells that are considered to be progenitor cells capable of both extended proliferation and differentiation into several cell phenotypes including osteoblasts, chondroblasts, fibroblasts, hematopoiesis-supporting stroma, and adipocytes (Friedenstein et al., 1974; Owen, 1988). In steady-state conditions in vivo, these cells are mostly in the $G_0$ stage of the cell cycle (Castro-Malaspina et al., 1980; Wang et al., 1990), and little is known about the factors that trigger them to proliferate and cause differentiation of their progeny. When BMSCs are cultured at low cell density, it has been demonstrated that each BMSC colony is derived from a single precursor cell termed "colony forming unit-fibroblastic (CFU-F) (Castro-Malaspina et al., 1980; Latsinik et al., 1986). However, these colonies contain cells of various morphologies and proliferation rates. Since the CFU-Fs present at the initiation of the culture are all exposed to the same tissue culture environment, the differences in growth rates must reflect each CFU-F's ability to respond to the growth-inducing factors present in the tissue culture medium, and, in turn, reflect the pattern of receptors that are present in that particular CFU-F. RTKs have been associated with regulation of proliferation in many cell systems. In this study, we examined if this class of receptors was involved in the regulation and the activity of the bone marrow stromal system. Our rationale was that by studying RTK expression in hBMSCs, it may be possible to identify the mechanisms underlying the differential proliferation and subsequent phenotypic capacities of this heterogeneous population of cells.

By amplifying RTK mRNA using degenerate oligonucleotides, followed by subcloning and sequencing, several RTKs were identified in multicolony-derived population of hBMSCs as well as in single colony-derived strains. Sequence analysis identified PDGF-R(β), EGF-R, FGF-R1, and Axl mRNA, suggesting that the ligands to these receptors may be important in regulation of BMSC proliferation.

The expression of PDGF-R(β), EGF-R, and FGF-R1

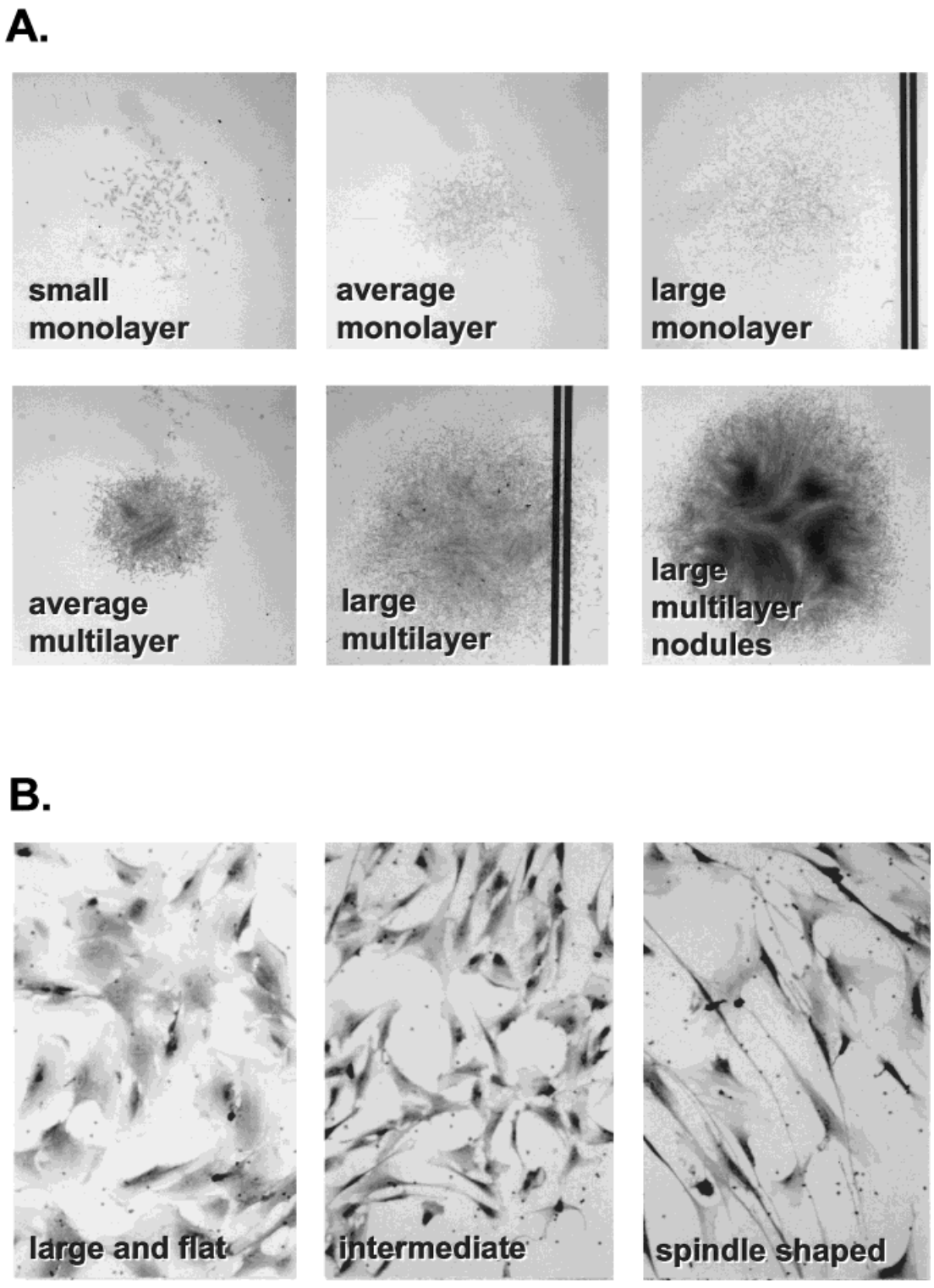


Fig. 5. Morphological variability of colonies of hBMSCs and their component cells. **A:** After 10–12 days of explantation of a single-cell suspension of bone marrow, there is a great deal of diversity in the size and structure of individual colonies of hBMSCs. Size ranges from small (with a few hundred cells), to average (from several hundred to 1,000 cells), to large (with thousands of cells), indicative of varying growth rates. Some remain as monolayers, while others form multilayers. Furthermore, some colonies develop nodular structures which stain positively with alizarin red (not shown) (small colony, ×20; all others, ×6.5). **B:** Within the colonies, there is also a variety of cell shapes, ranging from large, flat cells, through intermediate-sized cells, to a spindle-shaped morphology (×125).

TABLE 3. RTK expression in single colony-derived strains of hBMSCs determined by RT-PCR amplification[1]

| Clone no. | BF | Percent distribution | | | |
|---|---|---|---|---|---|
| | | PDGF-R | EGF-R | FGF-R | Axl |
| Bone-forming clonal strains | | | | | |
| 1 | 1.0 | 16.4 | 23.1 | 10.6 | 49.8 |
| 3 | 2.5 | 22.7 | 0.8 | 18.9 | 57.6 |
| 4 | 0.8 | 34.8 | 1.0 | 19.9 | 44.3 |
| 7 | 1.7 | 37.3 | 1.1 | 16.4 | 45.2 |
| 8 | 1.0 | 58.1 | 0.6 | 5.8 | 35.5 |
| 10 | 1.0 | 5.7 | 0.3 | 30.4 | 63.6 |
| 13 | 3.4 | 41.0 | 17.9 | 5.1 | 35.9 |
| 14 | 2.7 | 32.6 | 3.2 | 7.4 | 56.8 |
| 15 | 1.8 | 11.1 | 3.7 | 14.8 | 70.4 |
| 17 | 2.1 | 22.7 | 20.0 | 8.0 | 49.3 |
| Nonbone-forming clonal strains | | | | | |
| 2 | 0 | 25.6 | 35.4 | 5.9 | 32.9 |
| 5 | 0 | 42.2 | 5.5 | 14.3 | 38.0 |
| 6 | 0 | 24.8 | 31.2 | 9.8 | 34.2 |
| 9 | 0 | 22.3 | 0.9 | 23.4 | 53.5 |
| 11 | 0 | 24.8 | 3.0 | 16.8 | 55.4 |
| 12 | 0 | 7.8 | 2.6 | 25.2 | 64.3 |
| 16 | 0 | 0.0 | 34.2 | 15.8 | 50.0 |
| 18 | 0 | 7.8 | 11.8 | 11.8 | 68.6 |

[1]BF, bone formation.

by hBMSCs is consistent with what is known about the stimulation of the initial proliferation of BMSCs upon explantation. In human marrow cell cultures, the addition of PDGF-BB and/or EGF stimulates the proliferation of STRO-1-positive bone marrow stromal cells (Gronthos and Simmons, 1995). Recently, we showed that human and mouse BMSC colony formation is significantly inhibited by addition of anti-PDGF, anti-EGF, and anti-bFGF antibodies (Kuznetsov et al., 1997a), although in the present study, bFGF did not stimulate proliferation. However, it must be noted that the two previous studies focused on colony-forming efficiency rather than on proliferation of hBMSCs after passaging. Furthermore, we noted that with time in culture after passaging, hBMSCs do show a modest proliferative response to bFGF in serum-free conditions (data not shown). Others have also found that bFGF in the *presence of serum* stimulates the proliferation of a population of stromal cells that have an increased capacity to form bone in an in vivo transplantation assay (Martin et al., 1997). These results suggest that the effects of bFGF may depend on culture conditions, and the stage at which the cells are studied in vitro.

The PDGF-R β isoform was detected in all the hBMSC strains used in this study. PDGF-R contains two distinct subunits, α and β, which can combine to form three noncovalent dimeric forms, αα, αβ, and ββ. Only the β isoform appears to mediate ligand-induced changes in the cytoskeleton (Eriksson et al., 1992). Thus, the expression of PDGF-R (β) may also potentially reflect differences in cell morphology. The β isoform was previously detected in fibrous dysplasia and in mesenchyme-derived organs (Alman et al., 1995; Shinbrot et al., 1994), both of which may have cell populations related to the BMSCs used here. Though the α isoform was not detected in this study, it was detected previously in human osteosarcoma cells (Kose et al., 1996). PDGF-BB, a ligand of PDGF-R (β), significantly stimulated the proliferation of both a multicolony-derived population and single colony-derived strains in a dose-dependent manner. The tyrosine phosphorylation of intracellular molecules and the receptor itself was confirmed by Western blot analysis and immunoprecipitation. However, the levels of tyrosine phosphorylation and the effect of PDGF-BB on cell proliferation differed from one clonal strain to another.

Although highly variable, EGF-R was found in virtually all of the hBMSC strains used in this study. Previously, binding of EGF was localized to cells described as poorly differentiated preosteoblasts and osteoblasts in developing bone (Martineau-Doize et al., 1988).

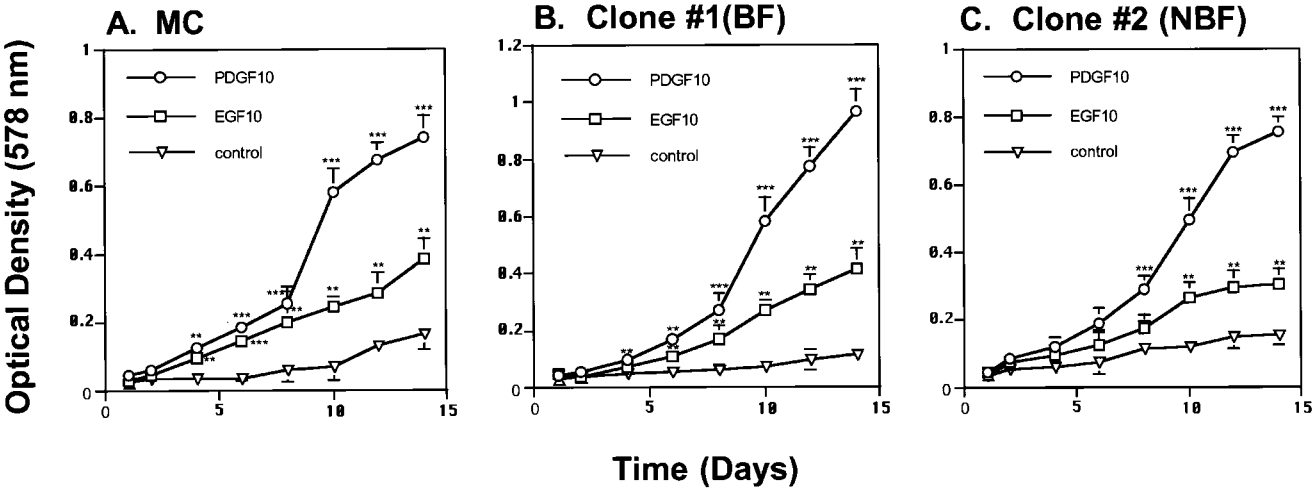


Fig. 6. Effect of PDGF-BB and EGF on the proliferation of hBMSCs with time in culture. Subconfluent cultures of a multicolony-derived strain (**A**) and two single colony-derived strains **B,** a bone-forming strain, clone 1, and **C,** a nonbone-forming strain, clone 2) were treated with 10 ng/ml of each growth factor. The cells were fixed with 1% glutaraldehyde at each time point, and stained with 0.02% crystal violet. The dye was eluted with 70% ethanol and the absorbency was measured at 578 nm. Values are the mean ± SD from three wells in a representative experiment. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. the culture without growth factor (control).

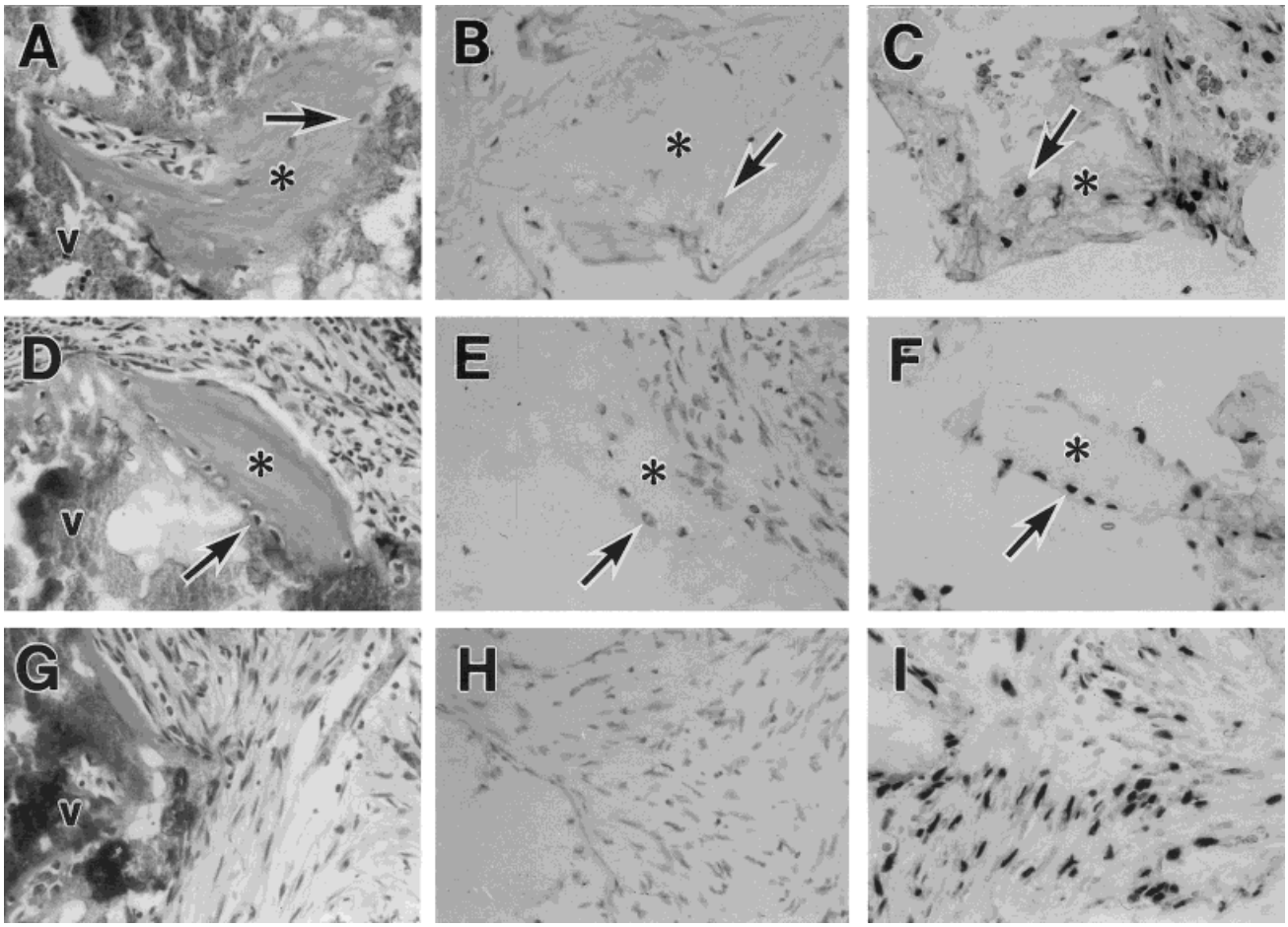


Fig. 7. Transplantation of hBMSCs into immunocompromised mice. One million cells each of a multicolony-derived strain (**A–C**) and two single colony-derived strains (**D–F,** clone 1, and **G–I,** clone 2) were implanted into immunocompromised mice with hydroxyapatite/tricalcium phosphate particles. After 8 weeks, the implants were retrieved and fixed with Bouin's solution. Five-micrometer-thick sections were stained with hematoxylin and eosin, immunostained with anti-human osteonectin antibody, or in situ hybridization for human specific *alu* sequences. New bone was formed only in the implants of a multicolony-derived strain (A) and a single colony-derived strain, clone 1 (D). The cells in the newly formed bone and surrounding fibrous tissue were immunoreactive with anti-human osteonectin antibody (B, E, and H), and these cells were also positive for *alu* sequences (C, F, and I). *Newly formed bone; arrows, young osteocytes embedded in bone matrix.

Based on the fact that exogenously added EGF stimulates cell proliferation in a number of osteoblastic (osteosarcoma and transformed) cells, and BMSC culture model systems (Bernier and Goltzman, 1992; Drake et al., 1994; Wise et al., 1992), the presence of receptor was implicated. Here, we show that EGF enhanced hBMSC proliferation as well as tyrosine phosphorylation of intracellular molecules and the receptor itself, and for the first time, we have demonstrated the expression of the actual receptor at the mRNA and protein level.

The present study is the first to identify FGF-R1 in human BMSCs, although its mRNA was expressed at very low levels in both multicolony-derived populations and single colony-derived strains. Also, bFGF did not significantly stimulate proliferation at any concentration in serum-free conditions. The pattern of FGF-Rs may be somewhat tissue-specific (Patstone et al., 1994), and it is now well-recognized that mutations of the FGF receptor family (R1, R2, and R3) cause a variety of skeletal anomalies, but most notably, different forms of craniosynostosis (Muenke and Schell, 1995). Although this spectrum of syndromes is most commonly associated with mutations in FGF-R2 (Apert's syndrome) and FGF-R3 (different forms of achondroplasia), mutations of FGF-R1 have been identified in cases of Crouzon's and Pfeiffer's syndromes. It is generally thought that mutations in these receptors cause increased osteogenesis, leading to premature closure of cranial sutures (i.e., FGF-Rs are negative regulators of bone formation) (Deng et al., 1996). Based on the studies presented here, it is not clear what role FGF-R1 plays in proliferation of and differentiation of hBMSCs. Although cDNA clones with FGF-R1 were easily detected, and mRNA was detected by RT-PCR in the different strains, this receptor did not appear to be functional, since bFGF did not stimulate cell proliferation in serum-free conditions. Further studies must be done in

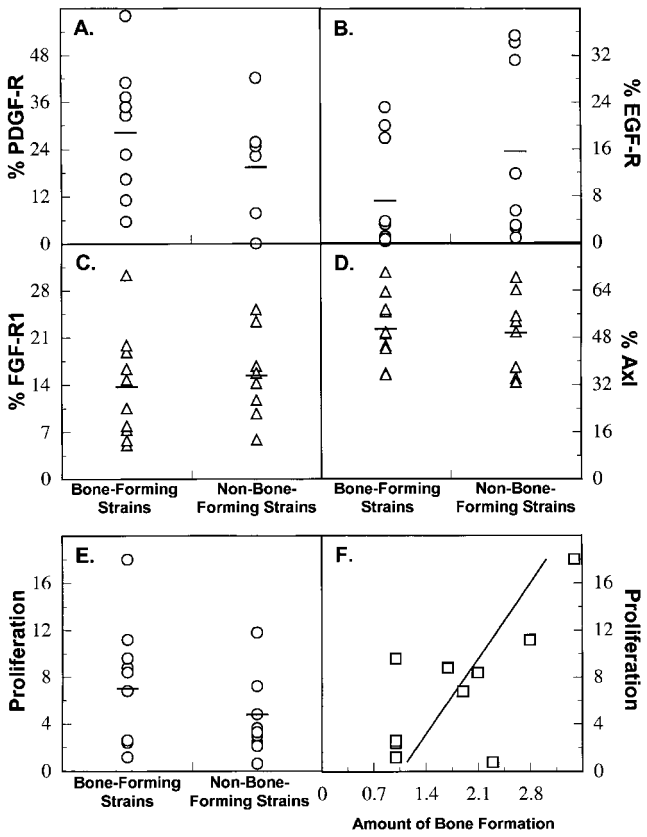


Fig. 8. Correlation of RTK expression and osteogenic capacity of single colony-derived strains of hBMSC. Single-colony hBMSC strains were analyzed for PDGF-R (β) (**A**), EGF-R (**B**), FGF-R1 (**C**), and Axl (**D**) by semiquantitative RT-PCR, and values were normalized to GapdH or osteonectin. The percent distribution among the four RTKs was then calculated for each strain, and the strains were grouped by their ability to form bone in the in vivo assay system. The groups were analyzed nonparametrically using Wilcoxon's sum of rank test. While a trend was noted for higher expression of PDGF-R in bone-forming strains and higher expression of EGF-R in nonbone-forming strains, the differences did not reach statistical significance ($P < 0.057$ for PDGF-R, and $P < 0.055$ for EGF-R). The two groups (one that formed bone and one that did not) were also compared using a measure of the proliferative capacity of the strains (**E**) and subjected to linear regression analysis of bone formation vs. proliferative capacity of the strains, found to positively correlated ($r^2 = 0.48$) (**F**).

order to clarify the role of this receptor and its ligand in bone metabolism.

In this study, Axl was found to be highly expressed by all hBMSC strains, matched only by PDGF-R in relative quantity. Axl is a transforming type of RTK discovered in acute myelogenous leukemia cells (Mark et al., 1994; O'Bryan et al., 1991), and also previously identified in the marrow stroma (Neubauer et al., 1994). Moreover, immunohistochemistry of human developing long bone with anti-Axl antibody indicated the restricted localization of Axl immunoreactivity in the periosteum and endosteum (Satomura and Gehron Robey, unpublished results). The proposed ligand for Axl is the product of Gas (growth arrest-specific) gene 6 (Varnum et al., 1995), which is vitamin K-dependent and homologous to protein S, an anticoagulatory protease regulator present in bone (Maillard et al., 1992). Recently, Gas6 was reported to have mitogenic and survival activities for serum-starved NIH-3T3 fibroblasts (Goruppi et al., 1996). However, since chemical amounts of either protein S or Gas6 are not available to us, it is not possible to speculate further on the role that Axl plays in the bone marrow stromal system.

Finally, we compared the pattern of RTK expression in a number of hBMSC populations derived from a single CFU-F that differed from one another by many parameters, including cell morphology, colony structure, growth rate, and osteogenic potential following in vivo transplantation. The results indicate that the pattern of RTK expression is highly variable from one single colony-derived hBMSC strain to another, irrespective of their capacity to form bone in the in vivo assay. While there is generally higher expression of PDGF-R in bone-forming strains, and relatively higher expression of EGF-R in nonbone-forming strains, there were exceptions to these generalities. In addition, we also noted that the response of different strains to PDGF-BB and EGF (as indicated by proliferation and generation of tyrosine-phosphorylated molecules) differed from one strain to another. These data suggest that the same growth factor ligand and its RTK may differentially regulate metabolic activity of different members of the BMSC family. Although the pattern of mRNA expression of these four particular RTKs was not indicative of the osteogenic capacity of single colony-derived hBMSC strains, further analysis of the intracellular pathways triggered by the ligands of these RTKs and further identification of other RTKs may provide a potentially new basis upon which different members of the BMSC family can be characterized, and may provide insight into the factors that control their proliferation and differentiation.

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